

Effects of probiotic bacteria and their genomic DNA on T_H1/T_H2 -cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects

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Received 31 August 2007; received in revised form 8 February 2008; accepted 12 February 2008

Abstract

Among the factors potentially involved in the increased prevalence of allergic diseases, modification of the intestinal flora or lack of microbial exposure during childhood has been proposed. T_H2 -cytokines increase the production of IgE and stimulate mast cells and eosinophils, whereas T_H1 -cytokines, such as IFN- γ , may suppress IgE synthesis and stimulate the expression of the secretory piece of IgA. Thus, a dysregulation in the expression of T_H1 - and T_H2 -cytokines may contribute to the initiation and maintenance of allergic diseases. Lactobacilli belonging to the natural intestinal microflora were reported to reduce the incidence of atopic dermatitis and the severity of allergic manifestations and to modulate T_H1/T_H2 responses. The mechanisms still remain to be elucidated.

We sought to assess the effect of different probiotics, *Lactobacillus rhamnosus* GG, *Lactobacillus gasseri* (PA16/8), *Bifidobacterium bifidum* (MP20/5), and *Bifidobacterium longum* (SP07/3), on the T_H1 and T_H2 responses of peripheral blood mononuclear cells (PBMCs) from healthy subjects and from patients with allergy against house dust mite to Staphylococcus enterotoxin A (SEA) and *Dermatophagoides pteronyssinus* (Dpt). To elucidate the molecular basis of these effects, the effects of bacterial genomic DNA were compared with the effects of viable bacteria. PBMCs from allergic patients and from healthy donors were incubated for 24 or 48 h, respectively, with or without SEA and Dpt allergens. The effects of preincubation with live probiotic bacteria and the effect of their genomic DNA, added simultaneously to cultures and incubated for 24 h, were assessed by measuring T_H1/T_H2 -cytokine production.

The tested live Gram-positive probiotic bacteria and their genomic DNA inhibited SEA- and Dpt-stimulated secretion of T_H2 -cytokines (IL-4 and IL-5) and enhanced the stimulation of IFN- γ . This effect was dose-dependent with a dosage-optimum, which was identical for all lactic acid producing bacteria (LAB) tested (10 bacteria per PBMC) and their DNA (75 ng/ml). Based on the maximal effects achieved with LAB and their DNA, more than 50% of the effects seem to be contributed by DNA. No significant effect was induced by the control, Gram-negative *Escherichia coli* TG1. Lactobacilli and bifidobacteria reduced SEA-stimulated IL-4 and IL-5 production more effectively in PBMCs from healthy subjects than from allergic patients. In contrast to this, inhibition of Dpt-stimulated IL-4- and IL-5-secretion was more pronounced in cells from allergic subjects. Compared with living LAB, bacterial

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DNA inhibited IL-4- and IL-5-secretion in a similar manner. SEA- and even more so Dpt-stimulated IFN- γ stimulation by living LAB was less pronounced in allergic than in healthy subjects, whereas IFN- γ stimulation by their DNA was more pronounced in allergic subjects.

The tested probiotic bacteria as well as their genomic DNA modulated the T_H1/T_H2 response to some allergens dose-dependently. DNA seems to contribute to 50% of the effect exerted by living bacteria in this *in vitro* model. The magnitude of the probiotic effects differed between healthy and allergic subjects. Whether the modulation found for the tested strains might be useful for the prevention and treatment of allergic diseases has to be assessed in clinical trials.

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Keywords: T_H1/T_H2-cytokines; Immune system; Allergy; Probiotics; Lactobacilli; Bifidobacteria

Introduction

The etiology of allergic diseases remains unclear, even if numerous studies indicate that genetic predisposition and environmental factors play a role. Additionally, gut-associated immunity and enteric bacteria may contribute to the pathogenesis of allergic diseases. Several studies suggest that T_H2-associated cytokines, particularly IL-4, IL-5, IL-9, and IL-13, play a pivotal role in pathogenesis by regulating the production of IgE and the activity of mast cells. Particularly IL-4, IL-5, and IL-13 were suggested to promote the development of allergic asthma (O'Byrne 2006). In contrast, IFN- γ , a T_H1 response-associated cytokine, is able to suppress IgE synthesis, as well as IgE-mediated allergies, e.g. allergic rhinitis, asthma and atopic dermatitis, have often been associated with defective IFN- γ expression (Behera et al. 2002; Holt et al. 1997; van der Velden et al. 2001).

The reasons for the increasing incidence of allergies and atopic diseases, which is no longer questioned, are also still largely unknown. According to the hygiene hypothesis, rare contact with antigens, particularly bacteria, during early childhood and/or an altered antigen pattern associated with alterations of the intestinal flora result in an imbalanced immunity and promote erroneous immune responses (Björkstén 1997; Holt et al. 1997; Isolauri et al. 2000; Martinez and Holt 1999; Strachan 2000).

Indeed, recent reports indicate that the administration of *Lactobacillus rhamnosus* GG (LGG) to mothers of children at high risk for atopic diseases before birth and to mothers or their infants after birth reduces the incidence of atopic eczema in these babies by half during a follow-up of more than 4 years (Kalliomäki et al. 2001, 2003). Moreover, administration of this strain and other lactic acid bacteria (LAB) reduced the severity of food allergy, atopic dermatitis, and allergic rhinitis in some (Isolauri et al. 2000; Majamaa and Isolauri 1997; Peng and Hsu 2005; Rosenfeldt et al. 2003; Wang et al. 2004; Weston et al. 2005), but not all studies (Fölster-Holst et al. 2006; Helin et al. 2002).

In attempts to understand the underlying mechanisms of these antiallergic effects, probiotic bacteria were shown to modulate the balance of the different T-helper cells (T_H1, T_H2, T_H3, Tr) and their associated cytokines and, especially, to shift immunity from a pro-allergic T_H2 response to a pro-defensive T_H1-response in response to antigens/allergens (Matsuzaki et al. 1998; Miettinen et al. 1998; Pochard et al. 2002; Shida et al. 1998).

Coincubation of peripheral blood mononuclear cells (PBMCs) from allergic subjects and healthy donors and coincubation with a variety of Gram-positive (potentially probiotic) LAB species *in vitro* inhibited allergen and superantigen (*Staphylococcus aureus* enterotoxine A, SEA) stimulated T_H2-cytokine release and stimulated the T_H1-cytokine response. In contrast, the Gram-negative bacteria investigated had no such effect (Hessle et al. 2000; Pochard et al. 2002).

It was further shown that not only whole living bacteria, but also some soluble factors secreted by them, including cell wall components such as peptidoglycans and lipoteichoic acid (Matsuguchi et al. 2003; Pena and Versalovic 2003), whole genomic DNA from *lactic acid bacteria* (Iliev et al. 2005; Lammers et al. 2003; Meier and Steuerwald 2005) and short DNA sequences containing unmethylated, phosphate-linked pairs of cytosine plus guanine (so-called CpG motifs) can modulate immune responses (Bohle et al. 1999; Helwig et al. 2006; Hessel et al. 2005; Kline et al. 1998; Klinman et al. 1996).

The purpose of the present study was to investigate whether the effects on T_H1/T_H2 balance, mainly seen with LGG and some lactobacilli, is a more general response elicited by (probiotic) lactobacilli and bifidobacteria and whether the responses to various species differ. For this purpose, lactobacilli and bifidobacteria strains were chosen and their species- and dose-dependent effects on T_H1/T_H2 immune responses of antigen/allergen-stimulated PBMCs from allergic and healthy subjects was examined and compared with that of LGG and a Gram-negative *E. coli* strain (TG1). These lactobacilli and bifidobacteria were demonstrated to exert

probiotic effects in previous studies performed by our group: in a double-blind study of healthy subjects the administration of these strains reduced the symptoms and duration of common cold episodes and increased the number and activity of CD4⁺ and CD8⁺ T-lymphocytes in the blood (de Vrese et al. 2005a, 2006).

It was a further goal to examine if and to what extent the presumed effects of these living bacteria could be explained by effects of their genomic DNA.

Material and methods

Bacterial culture

The bacterial strains used in this study were the following: *L. rhamnosus* GG, *Lactobacillus gasseri* PA 16/8, *Bifidobacterium bifidum* MF 20/5, *Bifidobacterium longum* SP 07/3, and L.gB.bB.1 (a mix of *L. gasseri*, *B. bifidum*, and *B. longum*) (de Vrese et al. 2005a,b). Using a 0.02% inoculum from stocks stored at -80°C in 30% glycerol, bifidobacteria and lactobacilli strains were grown anaerobically (The Modular Atmosphere Controlled System, MACS-VA500 Workstation with airlock, Don Whitley Scientific Limited, UK) in MRS broth medium (Lactobacilli MRS according to de Man, Rogosa, Sharpe; Merck, Darmstadt, Germany) supplemented with 0.05% L-cysteine, at 37°C for 16 h. Prior to harvesting and washing in PBS, serial dilutions of freshly prepared cultures were plated onto MRS-agar and cultured for enumeration. Colony forming units (CFU/ml) of probiotic bacterial groups were determined by plating serial 10-fold dilutions. Plates were anaerobically incubated at 37°C for 24–48 h. Bacteria were then washed three times with $1 \times$ phosphate buffered saline (PBS) and adjusted to final concentrations of 2×10^7 CFU/ml, and after dilution in complete medium were added to cell cultures. An aliquot of the bacterial samples were also stored at -80°C in MRS solution containing 30% glycerol for subsequent experiments. For all bacterial strains, standard growth curves were produced by plotting OD₆₀₀ or CFU of freshly prepared, serially diluted cultures vs. time. In order to calculate viable bacterial counts in freshly prepared cultures, these curves were fitted with logarithmic expressions, each of which yielded values of $>98.5\%$ (data not shown).

Gram-negative *E. coli* TG1 (product number BU-00035) was purchased from Maxim Biotech Inc., grown in Luria-Bertani Medium (LB) for 18 h at 37°C and harvested as described above.

Genomic DNA preparation from bacteria

Genomic DNA from overnight cultures of the probiotic bacteria was purified using the phenol–chloroform–

isoamyl alcohol extraction method (Sambrook and Russel 2001) with slight modifications. Briefly, after 12 h culture in 50 ml MRS at 37°C , the bacterial suspension was centrifuged at $5000 \times g$ for 10 min at 4°C . The supernatant was discarded and the pellet was washed twice with 10 ml PBS. After further centrifugation, the supernatant was discarded. The pellet was resuspended in 1.5 ml of freshly prepared lysis buffer (50 mM Tris–HCl (pH 8.0), 50 mM EDTA, 0.5% Tween-20, 0.5% TritonX-100, 20 mg/ml lysozyme, 40 $\mu\text{g/ml}$ mutanolysin, and 100 $\mu\text{g/ml}$ RNAase (Sigma-Aldrich, Taufkirchen, Germany)) and incubated at 37°C for 50 min to ensure efficient cell lysis and DNA release from the bacteria. One milliliter of 0.25 M EDTA was added and incubated for 5 min at room temperature and 400 μl sodium dodecyl sulfate (20% SDS) was then added and the mixture was incubated at 65°C until the solution cleared. Then, 20 μl proteinase K (20 mg/ml, Sigma-Aldrich, Taufkirchen, Germany) was added and incubated at 65°C for 15 min. The mixture was extracted with equal volumes of phenol, phenol–chloroform–isoamyl alcohol (25:24:1) and, finally, chloroform–isoamyl alcohol (24:1). This procedure was repeated until no interface was visible. Traces of phenol were removed with chloroform, and the salt concentration was adjusted by addition of 1:10 volume 3 M sodium acetate (pH 5.2). DNA was precipitated by adding two volumes of cold (-20°C) 96% ethanol, washed with 70% ethanol, and dissolved in 100 μl sterile double distilled water. The concentration and purity of all DNA preparations were determined by measuring OD₂₆₀ absorbance, OD_{260/280}, and OD_{260/230} ratios, respectively. Only DNAs with an OD_{260/280} ratio >1.8 and OD_{260/230} ≥ 2 were used. The quality of DNA was further analyzed by conventional gel electrophoresis, which showed only minor fragmentation due to the purification process. DNAs were purified from endotoxin with TritonX-114D and also assayed for lipopolysaccharide (LPS) content using the *Limulus* amoebocyte assay (QCL-1000, CAMBREX, Germany). LPS content was less than 0.01 U endotoxin/ μg DNA. To determine any bioactivity of LPS or other bacterial components in the DNA preparations, DNA was degraded by overnight incubation at 37°C with 5 mg/ml DNAase I (Sigma-Aldrich, Taufkirchen, Germany) in the presence of 5 mM MgCl₂. DNA degradation was confirmed by agarose gel electrophoresis with ethidium bromide staining. This degraded DNA (75 $\mu\text{g/ml}$) was tested according to intact DNA.

PBMC isolation and culture

Eight allergic patients with an average age of 40.13 ± 3.68 (mean $\pm$ S.E.M.) and five healthy donors with an average age of 30 ± 2.81 (mean $\pm$ S.E.M.)

(no significant difference in age) were recruited for the study in accordance with the Ethics Committee of the University of Kiel on the Use of Human Subjects in Research. Informed written consent was obtained from all subjects prior to their enrollment in this study. Venous blood was drawn from the donors into heparinized vacutainers and diluted 1:1 with 0.9% NaCl. PBMCs were isolated from heparinized diluted blood by density gradient centrifugation (1.077 g/ml) (Lymphoprep, AXIS-SHIELD PoC AS, Oslo, Norway). Cells were resuspended at 2×10^6 cells/ml in RPMI-1640 culture medium (Sigma, Munich, Germany) supplemented with 10% (v/v) heat-inactivated (56 °C, 1 h) fetal bovine serum, gentamycin (50 µg/ml) (Sigma), penicillin/streptomycin (1%), and sodium pyruvate solution (0.23 mmol/l) (Sigma) (called complete medium). All compounds were purchased endotoxin-free as determined by the Limulus amoebocyte assay (LAL). Cells were cultured at 37 °C in (1 ml) complete medium at concentrations of 2×10^6 cells/ml in 24-well plates (Nunc, Roskilde, Denmark).

PBMC stimulation with SEA and Dpt

During a 48 h culture period, PBMCs were incubated either in culture medium alone (basal values) or in medium with SEA (Sigma, 2 µg/ml) or Dpt (Citeq, 2 µg/ml). All experiments were performed in duplicate. After 48 h incubation, the culture medium was centrifuged at $1000 \times g$ for 20 min at 4 °C. Cell free supernatants were sterilized by passage through a 0.2 µm pore size filter (Millipore, Germany) and stored at –80 °C until use. Viability of cells was determined before and after incubation with bacteria by trypan blue exclusion.

PBMC preincubation with or without viable bacteria

PBMC from healthy versus allergic donors

PBMCs from five healthy donors and eight allergic patients were used to assess the effect of viable bacteria on basal cytokine secretion and SEA-or Dpt stimulated cytokine secretion. Four strains of LAB and one Gram-negative bacterium (*E. coli* TG1) were added to cultures (2×10^7 CFU/ml). Viability at the time of addition to cultures was determined by bacterial plate counts. Cells cultured with medium alone served as an unstimulated control. All experiments were performed in duplicate.

Dose–response experiments with viable bacteria

For dose–response experiments, PBMCs (2×10^6 ml^{–1}) from four healthy subjects and 5×10^4 , 5×10^5 , 2×10^6 , 5×10^6 , 2×10^7 , and 5×10^7 bacteria/ml in 200 µl/well each were coincubated in flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). This corresponded to 0.025, 0.25, 1, 2.5, 10, and 25 bacteria per

mononuclear cell. All experiments were performed in duplicate.

Since maximal inhibition was obtained with 2×10^7 CFU of bacteria/ml, corresponding to a ratio of 10:1 (bacteria to PBMCs) (Fig. 1), this concentration was used in further experiments in which the effects of viable bacteria were compared with those of genomic DNA and the differences between healthy and allergic subjects were assessed.

Time-dependency of effects by viable bacteria

For time-dependent experiments, PBMCs (2×10^6 ml^{–1}) from healthy donors ($n = 4$, aged 32.25 ± 2.17 years) were stimulated with SEA (2 µg/ml). Mononuclear cells were either preincubated for 1, 3, and 5 h with LGG (10:1 ratio) before SEA stimulus or stimulated simultaneously with the LGG strain and the superantigen, or LGG was added 1, 3, and 5 h after SEA stimulus and cell cultures were incubated over a period of 24, 48, and 72 h. At the indicated time points, cell-free supernatants were harvested and centrifuged. The supernatants were sterilized using 0.2 µm filters and stored at –80 °C until analysis of cytokines.

PBMC incubation with genomic DNA

PBMC from healthy versus allergic donors

PBMCs from five healthy and eight allergic subjects were used to assess the effect of genomic DNA on basal and SEA- as well as Dpt-stimulated cytokine secretion.

Dose–response experiments with genomic bacterial DNA

For dose–response experiments, 2×10^6 mononuclear cells and 5, 15, 25, 35, 45, 55, 65, 75, 85, 95, and 105 µg genomic DNA/ml in 200 µl/well each were coincubated for 24 h in flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). Since maximal inhibition was obtained with 75 µg DNA/ml (Fig. 1B), this concentration was used in further experiments in which differences in response to genomic DNA and the differences between healthy and allergic subjects were assessed. All experiments were performed in triplicate.

Cells were cultured in complete medium at concentrations of 2×10^6 cells/ml in 24-well plates (Nunc, Roskilde, Denmark) and, simultaneously, genomic DNA (75 µg/ml) of probiotic bacteria, LPS-free calf thymus DNA (Sigma, 75 µg/ml), SEA (2 µg/ml), or LPS from *E. coli* (100 ng/ml, Sigma, Munich, Germany) were added to cultures and incubated at 37 °C, 5% CO₂-humidified air for 24 h. After 24 h incubation, cell free supernatants were collected and centrifuged at $1000 \times g$ for 20 min at 4 °C, and sterilized by passage through a 0.2 µm pore size filter (Millipore, Germany) and stored at –80 °C in aliquots until analysis. Viability of cells was determined before and after incubation with genomic

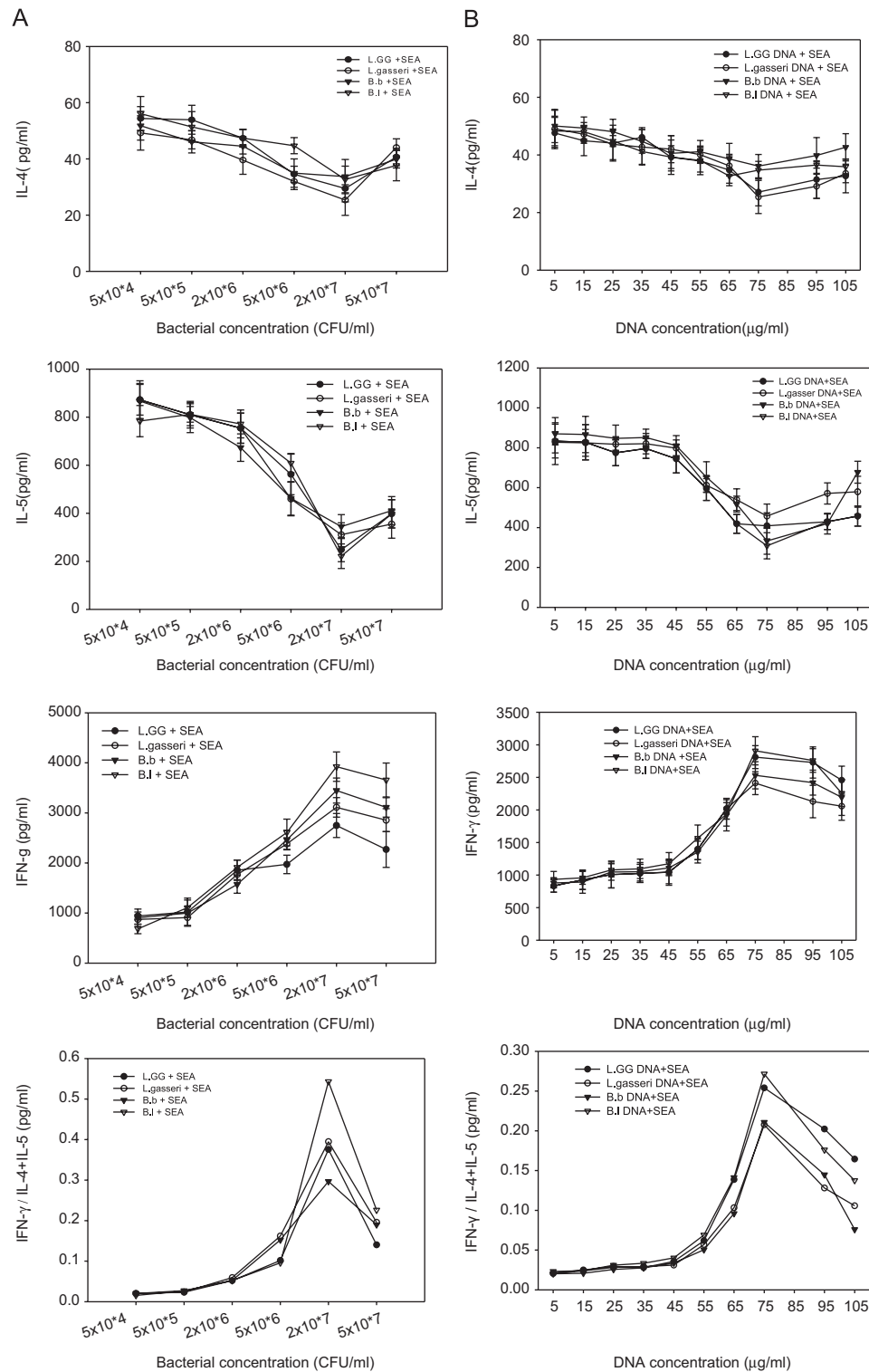


Fig. 1. Dose-dependent inhibitory effect of probiotic bacteria and their DNA on IL-4, IL-5, and IFN- γ /IL-4 + IL-5 ratio by SEA-stimulated PBMC from healthy subjects. (A) PBMC from four healthy donors (2×10^6 cells/ml) were cultured with four strains of live probiotic bacteria, LGG, *L. gasseri*, *B. b.*, *B. l.*, at concentrations ranging from 5×10^4 , 5×10^5 , 2×10^6 , 5×10^6 , 2×10^7 , and 5×10^7 corresponding to 0.025, 0.25, 1, 2.5, 10, and 25 bacteria per cell for 3 h before stimulation with 2μ g/ml of SEA. (B) PBMC from four healthy donors were cultured with different concentrations (5–105 μ g/ml) of bacterial genomic DNA in the presence or absence of SEA. After 48 and 24 h incubation with live bacteria and their DNA, respectively, IL-4, IL-5, and IFN- γ production were measured by specific ELISA. Data are expressed as mean $\pm$ S.E.M.

DNA by trypan blue exclusion. In all experiments, 95–98% of PBMCs were viable.

Time-dependency of genomic DNA-effects

For time-dependency experiments, PBMCs from four healthy donors (2×10^6 cells/ml and 200 μ l/well in flat-bottomed 96-well microtiter plates, Nunc, Roskilde, Denmark) were incubated with SEA (2 μ g/ml), 75 μ g/ml of LGG genomic DNA plus SEA and B. b plus SEA for 0, 24, 48, and 72 h. As controls medium alone, PBMC plus medium, and LPS plus SEA (100 ng/ml) were used. The concentrations of cytokines were assayed at intervals of 24 h and compared to those induced by LPS plus SEA.

Cytokine assessment

Levels of IL-4, IL-5, and IFN- γ were quantified in cell-free supernatants by means of specific ELISA (BD OptEIA™ set Human IL-4, 5, and IFN- γ , Heidelberg, Germany). The detection limit of the assay was 0.5 pg/ml for IL-4, 0.5 pg/ml for IL-5, and 1 pg/ml for IFN- γ . Optical density values of the samples were read at 450 and 570 nm on an ELISA plate reader (Molecular Devices, Munich, Germany). Experiments were repeated at least twice and run in triplicate.

Statistical analysis

Experimental data are expressed as mean $\pm$ S.E.M. and statistical analyses were performed with the unpaired *t*-test. The *p*-values of less than 0.05 were considered statistically significant.

Results

The characteristics of healthy and house dust mite allergic subjects are shown in Table 1.

Time- and dose-dependent responses

IL-4 and IL-5 showed maximal inhibition after 3 h preincubation of PBMCs with live bacteria followed by 48 h incubation with SEA (data not shown). This incubation time was used in further experiments. Results showed that lactobacilli and bifidobacteria had a non-linear dose-dependency effect on cytokine secretion. When PBMCs from healthy subjects were incubated with increasing concentrations of probiotic bacteria before adding SEA, IL-4, and IL-5 secretion decreased and IFN- γ and the $T_H1:T_H2$ -cytokine ratio increased, with a maximal effect observed at 2×10^7 CFU of bacteria/ml (Fig. 1A), corresponding to a bacteria-to-PBMC ratio of 10:1. Therefore, this dose was used in further experiments.

Table 1. Characterization of healthy and house dust mite allergic subjects

Subject	Age	Gender	RAST-tests (KU/l); ImmunoCap250				
			Total IgE ^a	d1 ^b	d2	h2	hx2

Healthy subjects								
1	36	M	32.7	0.03	–	–	–	
2	34	M	32.9	0.03	–	–	–	
3	21	M	35.8	0.03	–	–	–	
4	26	M	29.2	0.06				
5	33	M	11.0	0.02				
HDM allergic subjects								
1	51	M	14274	> 100				
2	41	M	10956	> 100	> 100	52.4	Positive	
3	47	M	2092	96.1				
4	26	M	9803	> 100	> 100	33.7		
5	33	M	12316	> 100				
6	34	F	171	< 0.35	< 0.35	1.43	Positive	
7	33	F	1278	5.54			Positive	
8	56	F	3914	21.7	23.2	4.86	Positive	

d1, *Dermatophagoides pteronyssinus*; d2, *Dermatophagoides farinae*; h2, Hollister-Stier-Labs; hx2, a mix of h2, d1, d2, and i6.

^aIgE-normal for adult: up to 100 kU/l.

^bd1, d2, h1, h2, and hx2 classes by ImmunoCap250: CAP-class 0: < 0.35 kU/l; CAP-class 1: 0.35–0.69 kU/l; CAP-class 2: 0.70–3.49 kU/l; CAP-class 3: 3.50–17.49 kU/l; CAP-class 4: 17.50–49.99 kU/l; CAP-class 5: 50.00–100.00 kU/l; CAP-class 6: > 100 kU/l.

Effects of antigens/allergens and viable bacteria

When PBMCs from healthy or house dust mite allergic subjects that had not been preincubated with bacteria were stimulated with the superantigen SEA or the specific allergen Dpt, an increase in IL-4, IL-5, and IFN- γ secretion was observed. However, this effect was small and not significant when PBMCs from healthy subjects were stimulated with Dpt (Fig. 2A).

Preincubation with lactobacilli and bifidobacteria before stimulation with antigen/allergen attenuated SEA- or Dpt-stimulated secretion, but not the unstimulated secretion of IL-4 and IL-5, and increased both unstimulated and SEA- and Dpt-stimulated IFN- γ secretion in PBMCs from allergic subjects (Fig. 2B).

In healthy subjects, similar effects were seen only in unstimulated and SEA-stimulated PBMCs but not in Dpt-treated cells (Fig. 2A).

No significant effects on the response of the Gram-negative control, *E. coli* TG1, were observed either in healthy or in allergic subjects (Fig. 2A and B).

When calculating the overall effect of preincubation with LAB, i.e. the ratio of the T_H1 (IFN- γ) and T_H2 -cytokine concentrations (sum of IL-4 and IL-5), a general shift towards the T_H1 response was observed in unstimulated and stimulated PBMCs from healthy and allergic subjects. However, this effect was again not seen

with *E. coli* TG1, and was not significant in all instances, particularly when PBMCs from healthy subjects were stimulated with Dpt.

Comparison of SEA- and Dpt-stimulation in PBMCs from healthy and house dust mite allergic subjects

In PBMCs from healthy subjects, preincubation with probiotic bacteria or the probiotic mix inhibited SEA-stimulated IL-4 and IL-5-secretion by 70% and 60%, respectively. In Dpt-stimulated PBMCs from healthy subjects, this inhibition was clearly less pronounced at about 5–15%. There was only one exception, in that preincubation with the probiotic mix resulted in a comparable inhibition of SEA- and Dpt-stimulated IL-5-secretion of around 60%. The increase in IFN- γ -secretion after preincubation with LAB was also higher in SEA-stimulated PBMCs (75–90%) than in Dpt-stimulated cells (50–65%). However, these differences were less pronounced (Fig. 3).

In contrast to this observation, probiotic bacteria inhibited SEA- and Dpt-stimulated IL-4- and IL-5-secretion in PBMCs from allergic subjects to a similar extent. Different from the observations in non-allergic subjects, inhibition in Dpt-stimulated PBMCs was somewhat more pronounced than in SEA-stimulated cells, and was, except for preincubation with the bacteria mix with approximately 40–60% inhibition, clearly higher than in Dpt-stimulated PBMCs of non-allergic subjects. The other way round, inhibition of the SEA-stimulated IL-4- and IL-5-secretion in bacteria-treated PBMCs of allergic subjects was always less than that observed for cells of non-allergic subjects (Fig. 3).

The effects of SEA- and Dpt-stimulation on the percent increase of IFN- γ -secretion in PBMCs of allergic subjects were similar for all probiotics used, were less after Dpt-stimulation and were less than that observed for PBMCs of healthy subjects (Fig. 3).

Effects of genomic DNA from Gram-positive lactobacilli and bifidobacteria

A second series of experiments examined the impact of bacterial DNA as compared to living bacteria on T_H1- and T_H2-cytokine secretion/production in PBMCs from healthy and allergic subjects. Again, stimulation of PBMCs from healthy subjects with SEA without DNA-preincubation caused a strong increase in cytokine secretion (IFN- γ , IL-4, and IL-5), but Dpt-stimulation caused only a small or no increase in cytokine secretion (Fig. 4). In PBMCs from allergic subjects, basal IL-4- and IL-5-secretion of unstimulated cells was somewhat higher and basal IFN- γ secretion was lower than in PBMCs from healthy subjects, and

stimulation with both SEA and Dpt led to a clear increase in cytokine secretion, which was more pronounced with SEA-stimulation.

Preincubation of PBMCs from healthy subjects with genomic DNA from all probiotic lactobacilli and bifidobacteria used in this study caused significantly decreased IL-4- and IL-5-secretion only in SEA-stimulated but not in Dpt-stimulated or unstimulated cells. IFN- γ -secretion was not affected by bacterial DNA in SEA-stimulated PBMCs from healthy subjects, but was increased slightly in unstimulated ($p < 0.05$) and Dpt-stimulated cells ($p > 0.05$; Fig. 4A).

Accordingly, the T_H1:T_H2-cytokine ratio was increased by bacterial DNA, meaning that there was a shift in the T_H1-direction, which was, however, not significant in Dpt-stimulated PBMCs from healthy subjects. This shift was mainly caused by the decrease in IL-4- and IL-5-secretion in SEA-stimulated cells and by the increase in IFN- γ secretion in un- and Dpt-stimulated PBMCs from healthy subjects (Fig. 4A).

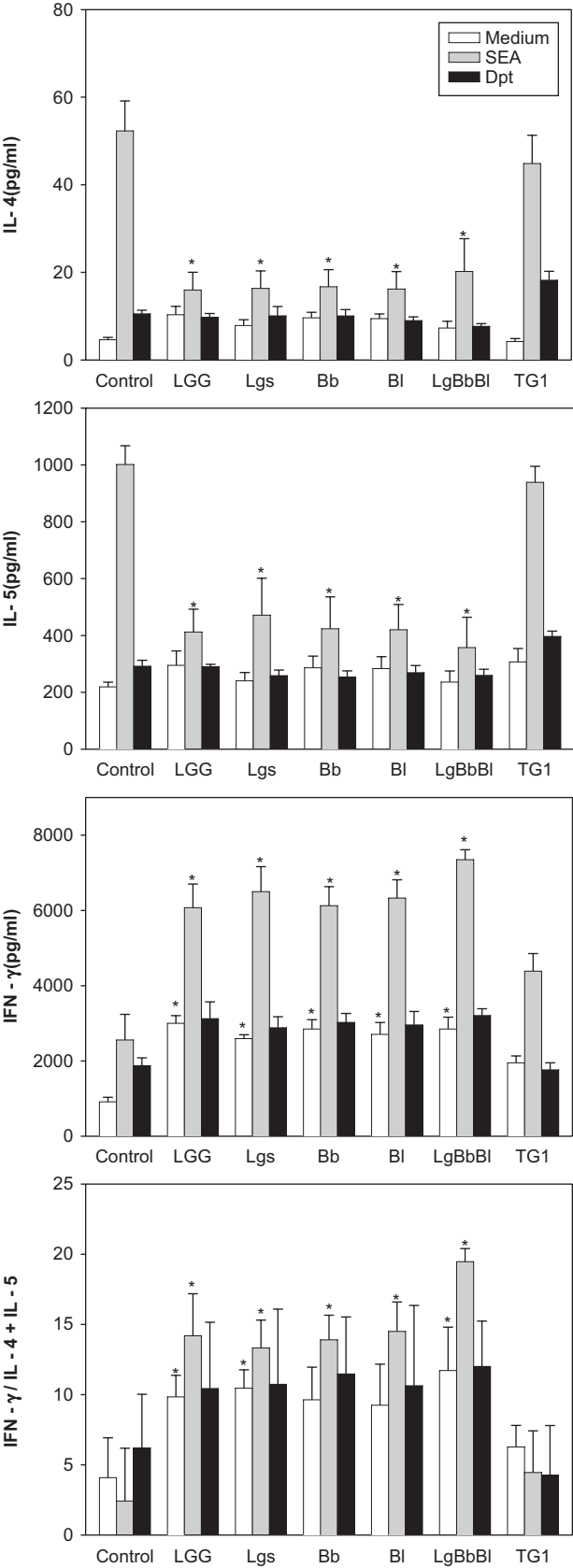
Experiments with PBMCs from house dust mite allergic subjects showed similar results. Pretreatment with genomic DNA from all lactobacilli and bifidobacteria tested resulted in significantly decreased IL-4- and IL-5-secretion from unstimulated and stimulated PBMCs. As a consequence, the T_H1:T_H2-cytokine ratio was significantly shifted in the T_H1 direction following DNA treatment. However, this shift was more pronounced in Dpt-stimulated PBMCs from allergic subjects than in those from healthy subjects (Fig. 4).

LPS, a cell wall component of Gram-negative bacteria used as control, had no effect on cytokine secretion or led to a small, but not significant, increase in IL-4- and IL-5-secretion in Dpt-stimulated PBMCs from healthy individuals and unstimulated cells from allergic subjects (Fig. 4).

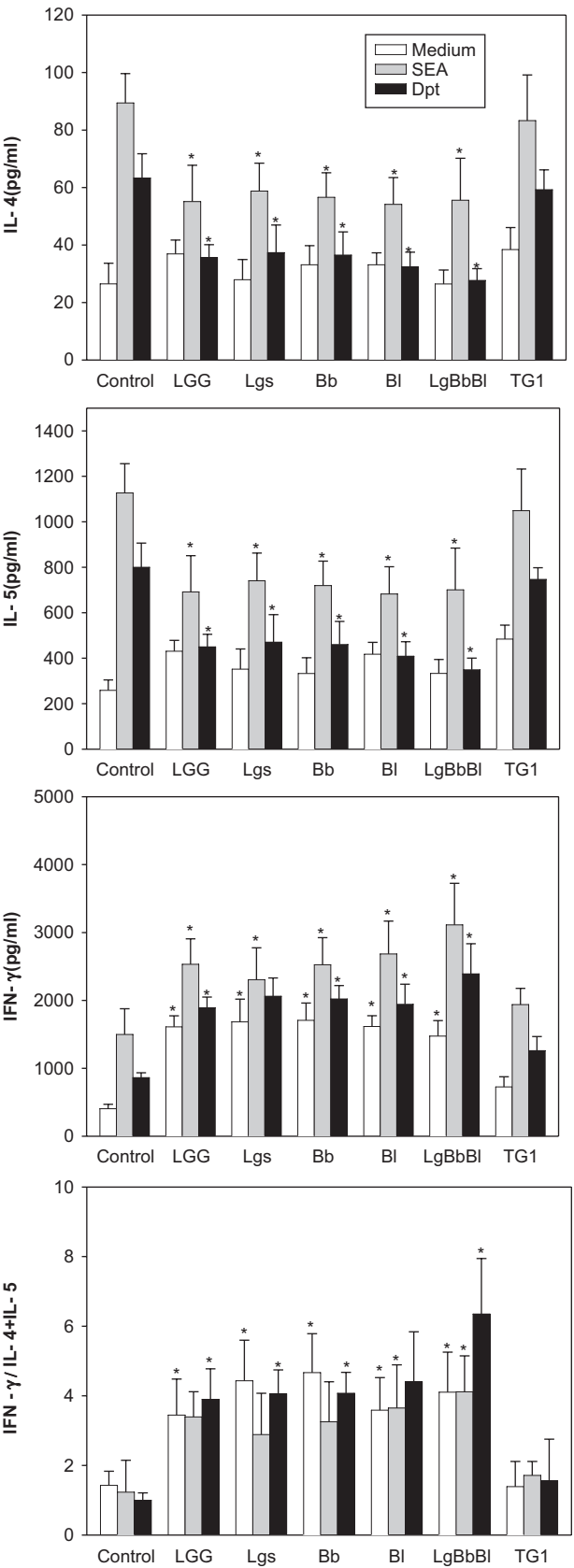
Dose- and time-dependency of DNA-effects

The effects of bacterial DNA on cytokine expression/secretion showed dose-dependencies similar to those of the corresponding living bacteria, i.e. IL-4- and IL-5-secretion decreased non-linearly, and IFN- γ secretion and the T_H1:T_H2-cytokine ratio increased non-linearly with increasing DNA concentration, with a minimum or maximum, respectively, at $\sim 75 \mu\text{g}$ DNA/ml (Fig. 1B). To prove that the decreased level of IFN- γ secretion was not a consequence of cell death due to high bacterial and DNA concentrations as well as IFN- γ , cell viability was verified by trypan blue exclusion. Microscopic examination of cells pre-treated with bacteria or DNA showed that viability was $> 95\%$ in all experiments, demonstrating that the reduction in IFN- γ was not due to cell death. For example, the viability of PBMCs after stimulation with 0, 75, 95, and 105 μg DNA B.l was

A



B



97.1 ± 3.1%; 96.8 ± 3.2%; 96.1 ± 1.8%; and 95.2 ± 8.7%, respectively.

When PBMCs from healthy subjects were incubated for various times with the cell-culture medium alone or together with SEA or SEA plus bacterial DNA, secretion of IL-4, IL-5, and IFN- γ increased non-linearly with increasing duration of incubation, reaching a maximum after 24 h incubation (Fig. 5).

Comparison between PBMCs from healthy and allergic subjects with respect to DNA-stimulated cytokine expression/secretion

If one compares the relative inhibition of IL-4- and IL-5-secretion or the relative stimulation of IFN- γ -secretion in SEA- and Dpt-stimulated PBMCs from healthy and allergic subjects rather than absolute cytokine concentrations, it becomes obvious that there was a strong inhibition of IL-4- and IL-5-secretion in SEA-stimulated PBMCs from healthy subjects, while in PBMCs from allergic subjects the strength of these effects were approximately equal after SEA- and Dpt-stimulation (Fig. 6).

When assessed as percentages, IFN- γ -secretion was not increased by bacterial DNA in SEA- and Dpt-stimulated PBMCs from healthy individuals, but was significantly stimulated in PBMCs from allergic subjects (Fig. 6).

Effects of lipopolysaccharides or other bacterial contaminants

In some experiments, LPS was used as control for bacterial DNA. It did not induce additional stimulation of cytokine secretion in unstimulated and stimulated PBMCs from healthy and allergic subjects (Figs. 4 and 5). DNA degraded with DNase I did not show any detectable inhibition of PBMC cytokine secretion below baseline levels.

Discussion

In this study, cocubation of live bacteria and their DNA with stimulated and unstimulated PBMCs was used as an *in vitro* model for testing the immunomodulatory effects of different LAB. This setting may seem to

be somewhat artificial, since the gastrointestinal microflora primarily comes into contact with epithelial cells and processes of dendritic cells *in vivo* (Winkler et al. 2007). Via M cells, however, bacteria can be forwarded to the subepithelial space, where they may come into contact with dendritic cells, macrophages and T-cells in the dome region (Winkler et al. 2007).

Although the PBMCs assay used in the present study was widely used for testing probiotic immunomodulatory properties (Cayzer et al. 2001; O'Mahony et al. 2006; Pochard et al. 2002), the results of such *in vitro* studies have rarely been linked to *in vivo* effects.

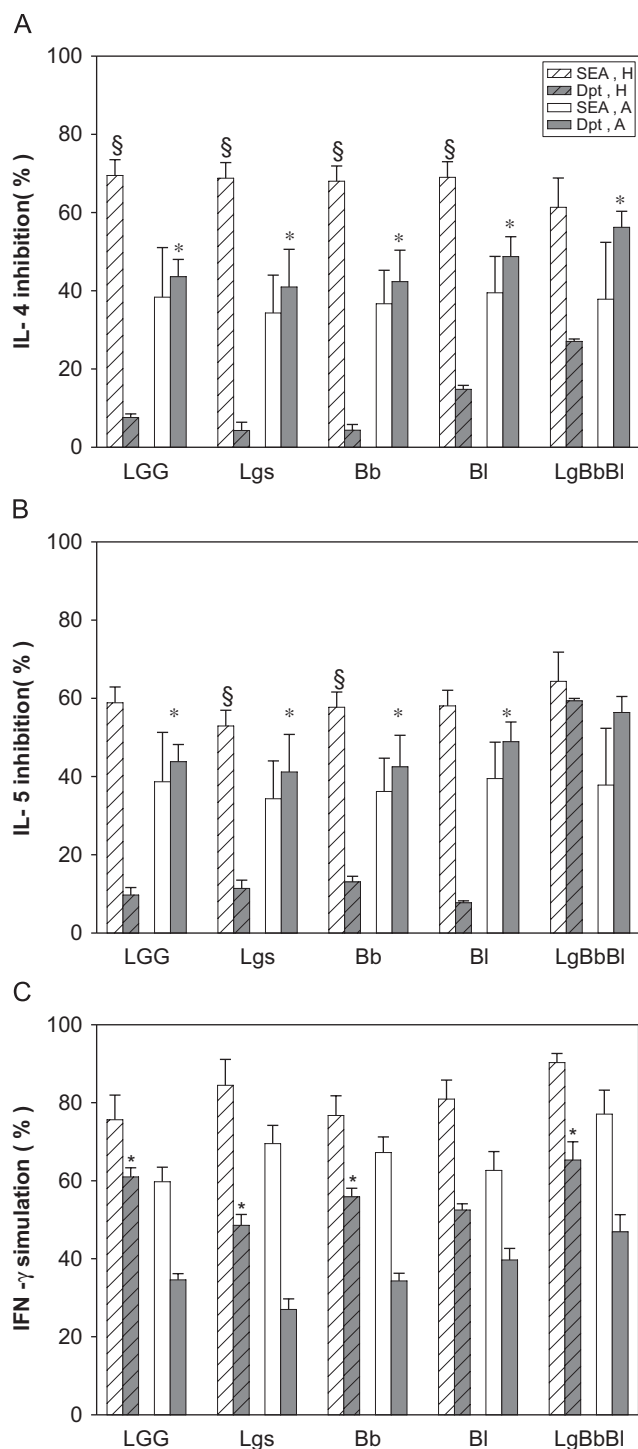
In a recent study, no significant difference between the cytokine responses of PBMCs after oral administration of probiotics to placebo was found, although the probiotics enhanced the secretion of IFN- γ , IL-10, and TNF- α by PBMCs *in vitro* (Flinterman et al. 2007). This, however, may be due to the small number of subjects investigated (seven children in the probiotic and six children in the placebo group). Foligne et al. (2007) found a correlation between the *in vitro* immunomodulatory properties of LAB and an *in vivo* murine colitis model. They concluded that the PBMC-based assay that they used, may serve as a useful primary indicator.

In this study, the probiotic lactobacilli and bifidobacteria tested increased the secretion of the T_H1-cytokine IFN- γ in PBMCs from healthy and allergic subjects and reversed the secretion of T_H2-cytokines (IL-4 and IL-5), which had been stimulated by pretreatment with an adequate antigen/allergen. Adequate in this context means that both the superantigen SEA and the specific allergen (Dpt) had significant effects in PBMCs from house dust mite allergic subjects, while only exposition to the superantigen significantly increased IL-4- and IL-5-secretion in PBMCs from healthy individuals, and this was largely inhibited by preincubation with the bacteria studied. Minor deviations from this outcome in individual experiments were within the range of expected variation. These IL-4 and IL-5 inhibitory effects were not observed with the Gram-negative *E. coli* TG1 strain, which was used as a control.

The mechanisms responsible for differential modulation of the T-Cell responsivity by Gram-positive (probiotic) lactobacilli and bifidobacteria and by Gram-negative bacteria such as *E. coli* were not further addressed in the present study.

Fig. 2. Cytokine modulation of PBMC without (medium) or with stimulation by SEA and Dpt. PBMCs from healthy (A, $n = 5$) and allergic (B, $n = 8$) subjects were preincubated for 3 h (at 2×10^6 cells/ml) with medium (control) or with four strains of probiotic bacteria, LgBbBI (a mix of probiotic bacteria) and one Gram-negative control bacterium (*E. coli* TG1) at a bacteria:cell ratio of 10:1 before either being stimulated with the SEA superantigen (2 μ g/ml), Dpt (2 μ g/ml) or not stimulated. IL-4, IL-5, and IFN- γ were quantified after incubation in supernatants by means of specific ELISA. The asterisks indicate a significant inhibition or stimulation ($*p \leq 0.05$, $**p \leq 0.01$) of T_H2- and T_H1-cytokine production in comparison with the control (without bacteria). Data are presented as mean $\pm$ S.E.M.

The finding that the effects of the Gram-positive probiotic LAB studied were very similar to each other indicates that modulation of the T-cell-associated immune response to an antigen/allergen from T_H2 towards T_H1 may be a general, more or less pronounced mechanism, which contributes to the probiotic potential of bifidobacteria and lactobacilli.



This interpretation is supported by a recently published report showing that other strains of *Lactobacillus plantarum*, *L. casei*, *L. rhamnosus*, or *Lactococcus lactis* promote the T_H1 response in SEA and Dpt-stimulated PBMCs of house mite allergic subjects (Pochard et al. 2002).

In another study, Gram-negative bacteria stimulated the secretion of the T_H2 -associated cytokine IL-10 in blood monocytes from healthy subjects, whereas all Gram-positive bacteria studied, including *L. plantarum*, *Bifidobacterium adolescentis*, and *Enterococcus faecalis* and a number of strains without established probiotic properties (*Clostridium perfringens*, *Corynebacterium minutissimum*, *Staphylococcus aureus*, and *Streptococcus mitis*) were potent inducers of IL-12 (Hessle et al. 2000), which is associated with the T_H1 response by activating IFN- γ secretion by T-cells. However, while administration of Gram-positive LGG to children with cow's milk allergy and atopic dermatitis stimulated IFN- γ secretion in their PBMCs, administration of a mixture of Gram-positive strains of *L. rhamnosus*, *Propionibacterium freudenreichii*, and *Bifidobacterium breve* modulated the immune response differently since it had no effect on the IFN- γ concentration, but led to an IL-4 response (Pohjavuori et al. 2004).

The observed shift from T_H2 - to T_H1 -cytokines may be interpreted as a pro-defensive immune response and a modulation of T-cell-associated cytokine production towards a lower allergenic risk.

A connection between the change in cytokine-secretion mediated by T-cells and the occurrence of allergic diseases has been described several times. Several experimental studies indicate that the initiation and maintenance of allergic diseases may be associated with a disruption of the T_H1/T_H2 -cytokine balance, i.e. a relative predominance of T_H2 -cytokines (IL-4, IL-5, IL-3, and IL-9) and insufficient secretion of T_H1 -cytokines (IFN- γ , IL-2) (van der Velden et al. 2001).

Fig. 3. Comparison of the effects of probiotic bacteria on IL-4, IL-5 (allergen-induced T_H2 -cytokines) and IFN- γ production by PBMC from healthy and allergic subjects. PBMC from five healthy (striped bars) and eight allergic (unstriped bars) subjects were preincubated for 3 h (at 2×10^6 cells/ml) with four strains of probiotic bacteria, LGG, *L. gasseri*, *B. b.*, *B. l.*, and LgBbBI (a mix of *L. gasseri*, *B. b.* and *B. l.*) at a bacteria:cell ratio of 10:1 (2×10^7 CFU/ml) before stimulation with SEA (2 μ g/ml, open and striped bars) or Dpt (2 μ g/ml, solid and striped bars). IL-4 (A), IL-5 (B), and IFN- γ (C) production were quantified after 48 h incubation by means of specific ELISA. Asterisk (*) indicates a significant difference in SEA-stimulated ($p < 0.05$) and (§) indicates a significant difference in Dpt-stimulated (§ $p < 0.05$) IL-4 and IL-5 production between healthy and allergic subjects. Data are expressed as mean $\pm$ S.E.M. H, healthy; A, allergic.

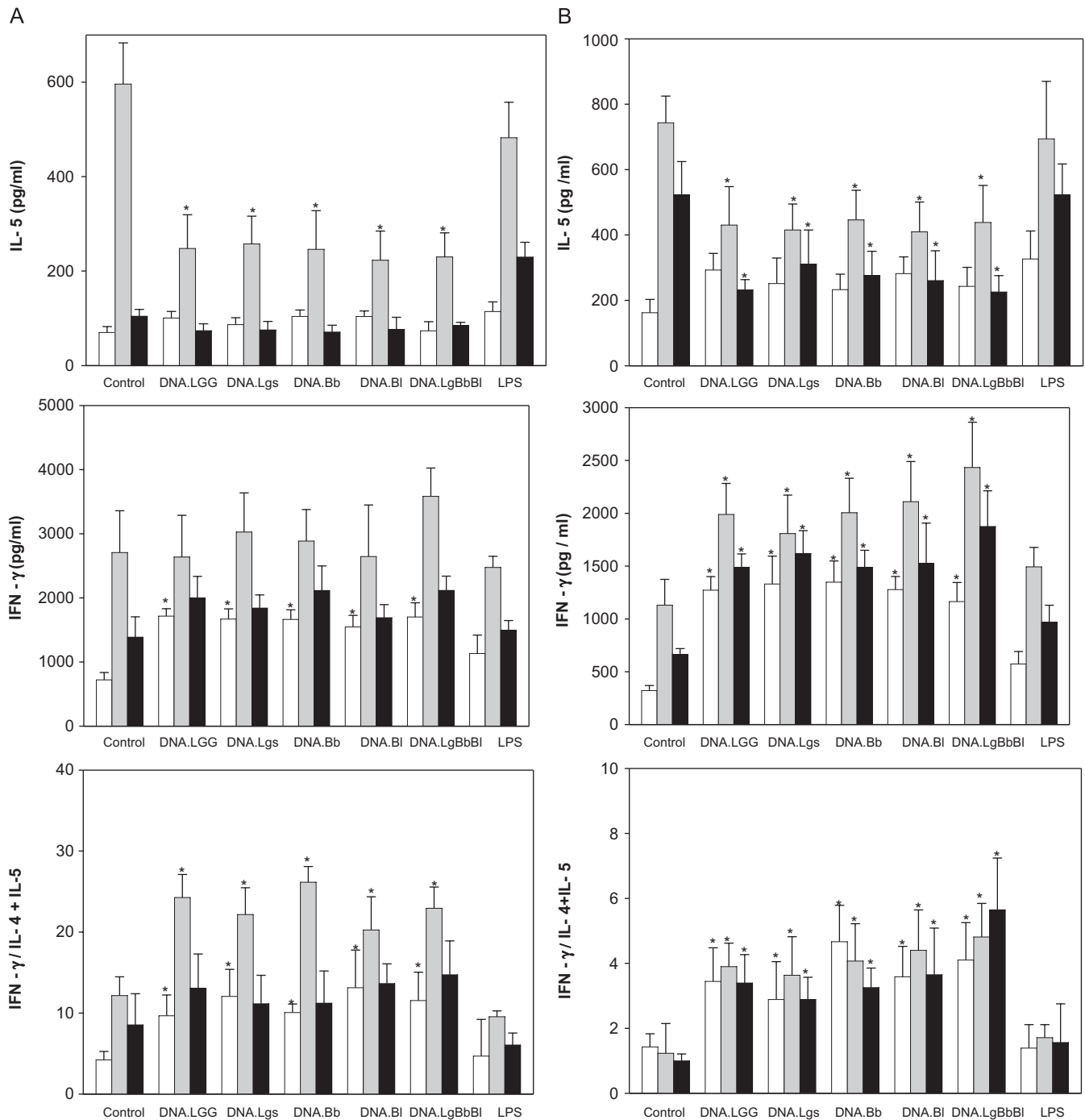


Fig. 4. Inhibitory effect of genomic DNA from probiotic bacteria on IL-4, IL-5, and IFN- γ production by SEA or Dpt-stimulated PBMC from healthy (A, $n = 5$) and allergic (B, $n = 8$) subjects. PBMC (2×10^6 cells/ml) were incubated for 24 h with or without (control) genomic DNA from four strains of probiotic bacteria, LGG, *L. gasseri*, *B. b.*, *B. l.* and LgBbBI (a mix of *L. gasseri*, *B. b.*, *B. l.*) at a concentration of 75 μ g/ml before stimulation with SEA (2 μ g/ml) or Dpt (2 μ g/ml). LPS was used at a concentration of 100 μ g/ml. IL-4, IL-5, and IFN- γ production were quantified after 24 h incubation by means of specific ELISA. Asterisks indicate a significant inhibition (* $p < 0.05$, ** $p < 0.01$) of cytokine production in comparison with control. Data are expressed as mean $\pm$ S.E.M.

Many of these studies used the model system used in the present study, i.e. human PBMCs stimulated with an antigen or a specific allergen (Martinez and Holt 1999; Majamaa and Isolauri 1997; Matsuzaki et al. 1998; Pochard et al. 2002).

PBMCs from allergic subjects demonstrated similar effects to PBMCs from non-allergic subjects, both unstimulated and following stimulation with the superantigen: (1) SEA-stimulated T_H1-cytokine (IFN- γ) production by PBMCs was increased by Gram-positive/

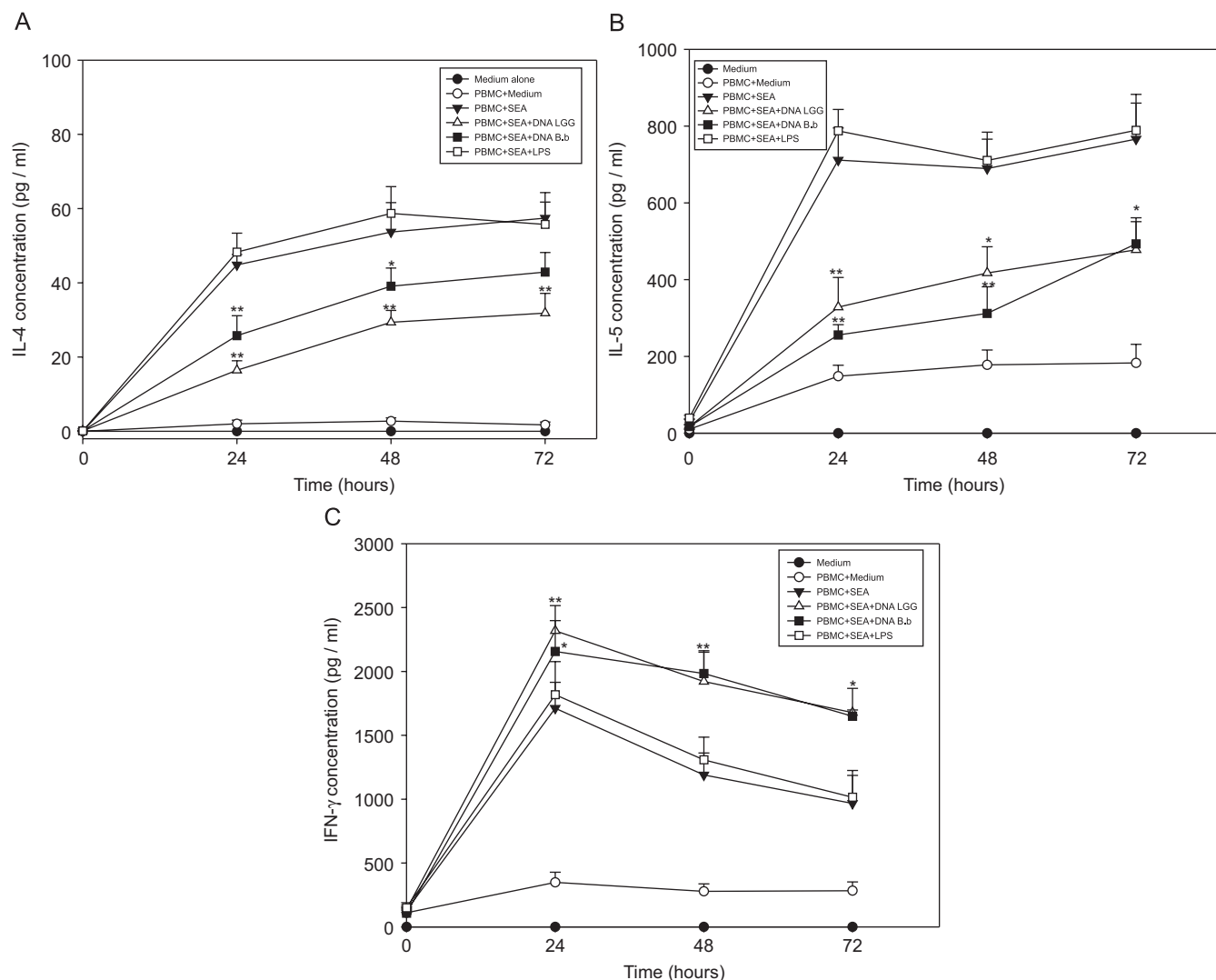


Fig. 5. Time-dependent effect of genomic DNA on IL-4 (A), IL-5 (B), and IFN- γ (C) production by PBMC from healthy subjects. PBMCs from four healthy donors were incubated with SEA (2 μ g/ml) and 75 μ g/ml of genomic DNA from LGG (open triangles) and *B. b.* (closed squares) or without DNA (closed triangles) for 0, 24, 48, and 72 h. As controls, medium alone (closed circles), medium plus PBMC (open circles), and medium plus PBMC plus LPS (100 ng/ml, open squares) were used. Concentrations of cytokines were compared to those induced by PBMC plus SEA. * $p < 0.05$, ** $p < 0.01$. Data are expressed as mean $\pm$ S.E.M.

probiotic LAB, but not by *E. coli*. (2) (SEA)-induced increased T_H2 -cytokine (IL-4, IL-5) production was decreased. However, the increase in IFN- γ secretion and the reduction in IL-5-secretion observed in bacteria-pretreated PBMCs from allergic patients were smaller compared with PBMCs from non-allergic subjects. This means that allergic subjects showed an overall more pronounced reduction in the SEA-stimulated T_H2 response after exposure to LAB.

In contrast, exposure to the specific allergen (Dpt) caused pronounced reactions in PBMCs from house mite allergics, while Dpt had only weak effects or none at all in PBMCs from healthy subjects.

In our study, not only live probiotic bacteria, but also their genomic DNA, inhibited IL-4 and IL-5 production

and stimulated IFN- γ secretion in SEA-stimulated PBMCs. The effects of living probiotic lactobacilli and bifidobacteria were not only comparable in qualitative terms with those of their respective DNA, but also in quantitative terms, since more than 50% of the effect's magnitude of the bacteria could be explained by the effect of DNA. Such a comparison can be made as both bacteria and their respective DNA were tested using incubation conditions that assured maximal effects, based on pre-trial evaluation time- and dose-dependency (Figs. 2, 5 and 6). Other comparisons between live probiotic bacteria and their DNA as well as observations using heat-killed bacteria with intact DNA fit with these results: in a recent study performed by another group, some probiotic bacteria and their genomic DNA

increased IL-12 and IFN- γ production by PBMCs (Medina et al. 2007), and previous studies indicate that heat-killed probiotic bacteria reduced IL-4 and IL-5 production by SEA-stimulated PBMCs (Pochard et al. 2002) and live probiotic bacteria induced IFN- γ secretion by *Staphylococcus aureus* enterotoxin B (SEB)-stimulated PBMC (Prescott et al. 2005). Additionally, LAB can highly reduce the capacity of allergen-specific human T-cells to produce T_H2-cytokines (IL-4, IL-5),

but, in contrast, largely favored the production of IFN- γ (Pochard et al. 2005).

LPS, a component of the cell walls of Gram-negative bacteria, which was examined in the present study, could not elicit the effects described here for living bacteria and their DNA. LPS, however, is known to be a poor inducer of IFN- γ secretion in PBMCs (Müller-Alouf et al. 1996).

In the present study, the inhibitory effect of probiotic bacteria and their DNA on IL-4 and IL-5 production as well as the stimulation of IFN- γ secretion were dose-dependent (Figs. 2 and 5).

Based on the comparison of maximal inhibition of IL-4 and IL-5 and the maximal stimulation achieved by viable LAB (Fig. 2) and their DNA (Fig. 5), more than 50% of the effects of LAB can be attributed to DNA.

At least part of the DNA-effects are mediated by CpG motifs via Toll-like receptor 9 (TLR9) (Winkler et al. 2007). CpG ODNs are oligodeoxynucleotides containing one or more unmethylated CpG dinucleotides in specific base sequences. CpG ODNs are known to trigger T_H1 responses by increasing IFN- γ secretion (Hussain et al. 2002; Kline et al. 1998) and to inhibit established T_H2 responses *in vitro* (Kitagaki et al. 2002) and *in vivo* (Broide et al. 2001). This effect is suggested to be mediated either through IFN- γ and IL-10 (Kitagaki et al. 2002) or independently through T_H1-cytokine expression (IFN- γ and IL-12) (Kline et al. 1999).

In our study, both effects showed an optimum at a bacteria/PBMC ratio of 10:1, corresponding to ~75 μ g DNA/ml, and this effect was reversed at higher concentrations. This could indicate that 10 bacteria per PBMC enable maximal signaling. This might be due to the average number of PAMP receptors on the cell. However, this cannot explain the inversion of these effects, i.e. the increase in IL-4 and IL-5 and the decrease in IFN- γ , at higher concentrations. Therefore,

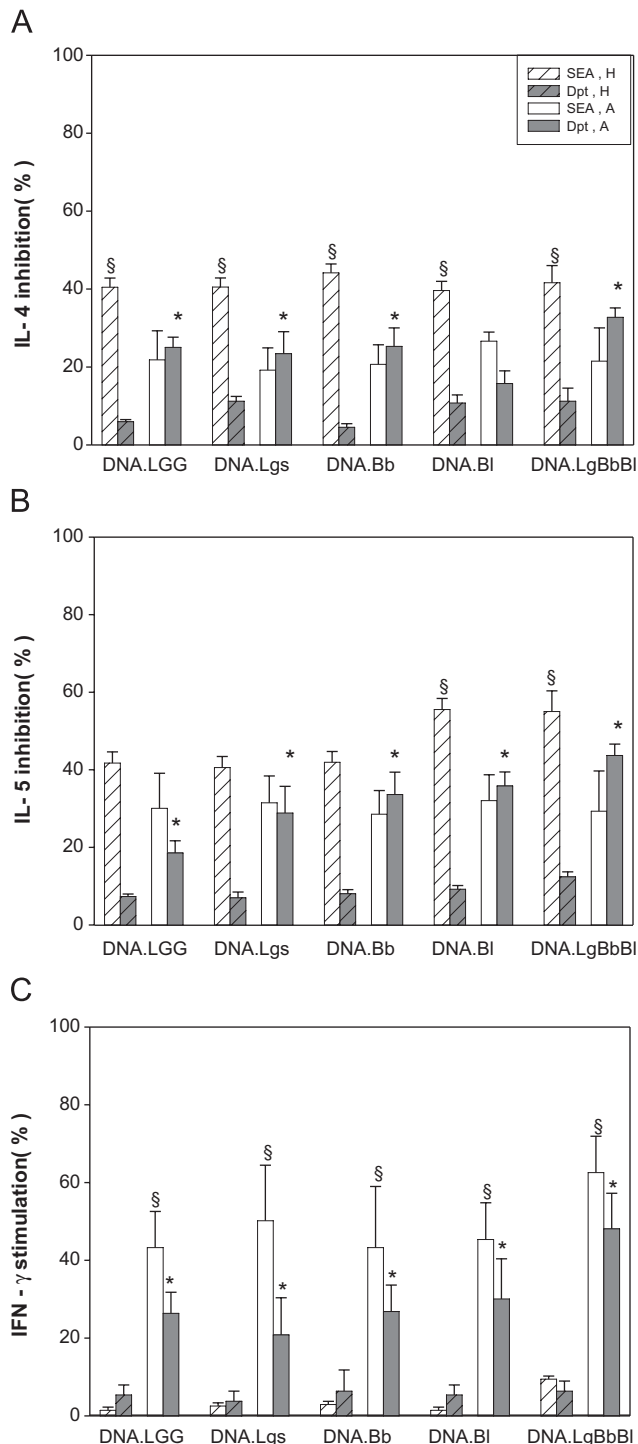


Fig. 6. The inhibitory effect of DNA from probiotic bacteria on IL-4, IL-5 (allergen-induced T_H2-cytokines), and IFN- γ production by PBMC from healthy and allergic subjects. The inhibition by probiotic DNA is expressed as the percentage of cytokine concentration found in controls without probiotic DNA. PBMC (2×10^6 cells/ml) from five healthy (striped bars) and eight allergic (unstriped bars) subjects were incubated for 24 h with genomic DNA from four strains of probiotic bacteria, LGG, *L. gasseri*, *B. b.*, *B. l.* and LgBbBI (a mix of *L. gasseri*, *B. b.*, *B. l.*), at a concentration of 75 μ g/ml before stimulation with SEA (2 μ g/ml, open and striped bars) or Dpt (2 μ g/ml, solid and striped bars). IL-4 (A), IL-5 (B), and IFN- γ (C) production were quantified after 24 h incubation by means of specific ELISA. Asterisk (*) indicates a significant difference in SEA-stimulated (* p <0.05) and (\$) indicates a significant difference in Dpt-stimulated (§ p <0.05) IL-4 and IL-5 production between healthy and allergic subjects. Data are expressed as mean $\pm$ S.E.M. H, healthy; A, allergic.

other or additional mechanisms have to be taken into account.

In a study using mice splenocytes (Kitagaki et al. 2002), CpG ODNs induced expression of IFN- γ and inhibited IL-5 production. The authors suggested that IFN- γ was an important (but not the sole) factor in the concentration-dependent inhibition of antigen-induced IL-5 production. At higher concentrations of CpG ODNs, however, IFN- γ stimulation was found to be decreased. The authors suggested that this was due to IL-12 mediated, CpG ODN-induced IL-10-secretion.

Previous data together with results from the present study suggest that with increasing bacterial density and/or increasing concentration of DNA containing CpG ODNs, IFN- γ concentration increases and, partly mediated by IFN- γ , IL-4, and IL-5 concentrations decrease. Above the optimal concentration (i.e. a bacteria/PBMC ratio of 10:1, corresponding to $\sim 75 \mu\text{g}$ DNA/ml), IFN- γ overproduction is down-regulated to prevent an excessive T_H response, and the IFN- γ -mediated inhibition of antigen-stimulated IL-4- and IL-5-secretion is reduced. Whether this really is mediated via IL-12 and IL-10 and by which mechanism remains to be clarified, e.g. by knock-down experiments.

In conclusion, the Gram-positive (probiotic) lactobacilli and bifidobacteria tested, as well as their genomic DNA, dose-dependently modulated the T_H1/T_H2 response to allergens. DNA seemed to contribute to about 50% of the immunomodulatory effects exerted by live bacteria. Based on the limitations of this *in vitro* model, extrapolations to *in vivo* effects must be considered with caution. Therefore, clinical trial assessment is required to elucidate whether this modulation might be useful in the prevention and treatment of allergic diseases, and whether *in vitro* differences between species or even strains of bacteria are predictive of their clinical efficacy.

Acknowledgments

We thank N. Roesch for excellent technical assistance with the MACS-VA500 Workstation and Gaby Kolfenbach from the RAST Laboratory of Clinical Dermatology of University of Kiel for performing the RAST tests.

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